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Pharmacodynamics, Tissue Distribution, Toxicity Studies and Antitumor Efficacy of the Vascular Targeting Fusion Toxin VEGF₁₂₁/rGel

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Abstract

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As a part of an ongoing assessment of its mechanism of action, we evaluated the *in vivo* pharmacokinetics, tissue distribution, toxicity and antitumor efficacy of VEGF₁₂₁/rGel, a novel fusion protein. Pharmacokinetic studies showed that VEGF₁₂₁/rGel cleared from the circulation in a biphasic manner with calculated half-lives of 0.3 and 6 hours for the alpha and beta phases, respectively. Pharmacokinetic evaluation of ⁶⁴Cu-DOTA-VEGF₁₂₁/rGel showed relatively high blood retention 30 min after injection (26.6 ± 1.73 %ID/g), dropping to 11.8 ± 2.83 % and 0.82 ± 0.11 % ID/g at 60 and 240 minutes post injection, respectively. Tissue uptake studies showed that kidneys, liver and tumor had the highest drug concentrations 48 hrs after administration. The maximum tolerated dose (MTD), based on a QOD X5 i.v. administration schedule, was found to be 18 mg/kg with an LD₅₀ of 25 mg/kg. Treatment of BALB/c mice with VEGF₁₂₁/rGel at doses up to the MTD caused no alterations in hematologic parameters. However, AST and ALT parameters increased in a dose-related manner. The no-observable-adverse-effect-level (NOAEL) was determined to be 20% of the MTD (3.6 mg/kg). VEGF₁₂₁/rGel treatment of mice bearing orthotopically-placed MDA-MB-231 breast tumors caused increased vascular permeability of tumor tissue by 53% compared to saline-treated controls. Immunohistochemical analysis showed significant tumor hypoxia and necrosis as a consequence of vascular damage. In summary, VEGF₁₂₁/rGel appears to be an effective therapeutic agent causing focused damage to tumor vasculature with minimal toxic effects to normal organs. This agent appears to be an excellent candidate for further clinical development.

Graphical Abstract (for review)

Keywords

Angiogenesis; Necrosis; Pharmacokinetics; Toxicology; VEGF; Vascular permeability

1. Introduction

Many antineoplastic agents effectively target cancer cells but they possess limited selectivity and are highly toxic to normal cells, resulting in low therapeutic indices. Numerous groups have developed a targeted therapy approach employing antibodies or ligands, to specifically bind to cell surface receptors overexpressed in cancer. This approach is further enhanced by highly cytotoxic payloads conjugated or fused to these cell-targeting molecules, which is internalized directly into the targeted cell, sparing normal tissues. Antibody-drug conjugates (ADCs) have gained considerable attention recently based on several promising clinical trial results demonstrating efficacy against a number of tumor targets [1;2]. Highly cytotoxic protein payloads have also been developed including gelonin, pseudomonas exotoxin,

diphtheria toxin, saporin, and ricin A-chain [3–6]. Currently, one growth factor/toxin (denileukin diftitox) is approved for clinical use by the FDA [7].

Vascular endothelial growth factor (VEGF) is one of several cytokines intimately involved in the growth and development of normal and tumor vasculature. The smallest isoform in the VEGF-A family of cytokines, VEGF₁₂₁ binds to the receptors - Flt-1/FLT-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) [8]. VEGF₁₂₁ has been shown to contain the full biological activity of the larger variants. Both VEGFR-1 and VEGFR-2 are over-expressed on the endothelium of tumor vasculature including lung, brain, breast, colon, prostate, skin and ovarian cancers [9;10]. In contrast, these receptors are almost undetectable by immunohistochemistry in normal tissues other than pancreas and kidneys.

We have previously demonstrated that the growth factor fusion toxin VEGF₁₂₁/rGel effectively targets neovasculature overexpressing VEGFR-2 and destroys tumor neovasculature in solid tumors, reduces breast cancer metastatic spread and vascularization of metastasis in lungs. Furthermore, through a unique mechanism, this agent was found to prevent tumor growth in bone in experimental bone metastasis models. Systemic or intraocular VEGF₁₂₁/rGel administration caused significant regression of retinal and choroidal neovascularization in several models of ocular disease indicating the applicability of this approach in non-oncology settings [11–16].

Tumor VEGFR-2 expression levels assessed through non-invasive methods represent an opportunity to record tumor angiogenesis status, the targeting efficiency of cancer therapeutics, the ability to select patients that are likely to respond, and to monitor treatment efficacy. We initiated studies with ⁶⁴Cu-DOTA-VEGF₁₂₁/rGel to calibrate the utility of trace levels of VEGF₁₂₁/rGel for this purpose. Noninvasive bioluminescence imaging (BLI), magnetic resonance imaging (MRI), and positron emission tomography (PET) imaging were shown to be effective in tracking and quantifying VEGF₁₂₁/rGel inhibition of orthotopic glioblastoma growth through specific tumor vasculature targeting. PET clearly demonstrated VEGFR-specific tumor uptake of ⁶⁴Cu-DOTA-VEGF₁₂₁/rGel and favorable pharmacokinetics for radionuclide imaging and therapy, suggesting that labeled VEGF₁₂₁/rGel administered in trace amounts may be a useful guide in treatment monitoring and patient stratification [17]. While ⁶⁴Cu-labeled agents have proven useful clinically in PET studies [18;19], the presence of ⁶⁴Cu and DOTA may alter the distribution profile of the labeled compound and compromise information gained by this approach.

As a part of our ongoing pre-clinical assessment of the suitability of the VEGF₁₂₁/rGel fusion construct we examined the pharmacokinetics and tissue distribution of this novel agent. This provided a rationale for design of an i.v. dosing schedule (QOD X5) which was explored to set the MTD studies and toxicity assessment for VEGF₁₂₁/rGel. Finally, we examined the ability of VEGF₁₂₁/rGel to impact tumor hypoxia and vascular integrity of normal tissues and tumor xenografts and the mechanism of cell death.

2. Materials and Methods

2.1 Purification of VEGF₁₂₁/rGel

Construction, bacterial expression and purification of VEGF₁₂₁/rGel was essentially the same as described [14]. The purity of the final product was assessed via RP-HPLC and SDS-PAGE and found to be >90% pure. Endotoxin content was found to be < 2 EU/mg. The VEGF₁₂₁/rGel was concentrated and stored in sterile PBS at -20°C.

2.2 Pharmacokinetic Studies of VEGF₁₂₁/rGel in Mice

Twenty-four mice (6–8 weeks old, BALB/c) were injected (i.v., tail vein) with 0.1 mg of pre-clinical grade VEGF₁₂₁/rGel (1 mg/ml). Three mice at each time point were sacrificed at 5, 15, 30, 60, 120 minutes and at 4, 8, and 24 hours after administration. Blood samples (cardiac puncture) were withdrawn immediately after cervical dislocation and the samples were centrifuged (2 min @ 12,000 × g). Serum samples were removed and frozen at -80°C until analysis. Quantitative ELISA for VEGF₁₂₁/rGel was performed on each sample in triplicate utilizing the assay [16]. The mean concentrations for all three animals at each time point were subjected to pharmacokinetic analysis (Rstrip, from MicroMath, Inc).

2.3 Tissue Distribution of ¹²⁵I-VEGF₁₂₁/rGel

Nude mice bearing orthotopic MDA-MB-231 tumors were injected (i.v.) with 1 µCi of ¹²⁵I-labeled VEGF₁₂₁/rGel, prepared as described [20]. At 24 and 48 hours post injection (p.i.), the mice were sacrificed and tissues and blood were harvested, wet weighed and counted to determine the total radioactivity in a γ-counter (Packard, Model 5360; Packard, Inc, Meriden, CT).

2.4 Biodistribution of ⁶⁴Cu-DOTA-VEGF₁₂₁/rGel in BALB/c mice

DOTA-VEGF₁₂₁/rGel conjugate was synthesized according to our previously reported procedure with some modifications [17]. Briefly, VEGF₁₂₁/rGel was dissolved in PBS at a concentration of 0.6 mg/ml. 800 µl of VEGF₁₂₁/rGel was further buffered by 200 µl of borate buffer (200 mM, pH 8.5). To the buffered protein, excess amount of NHS ester of 1,4,7,10-tetraazadodecane-N,N',N'',N'''-tetraacetic acid (DOTA) (Macrocyclics, Inc. Dallas, TX) dissolved in DMSO was added (10 mg/ml, 1.6 µl). Molar reaction ratio of DOTA to VEGF₁₂₁/rGel was 10:1. The solution was incubated at room temperature for 60 min. DOTA-VEGF₁₂₁/rGel was isolated from the free ligand using a PD-10 column (GE Healthcare) equilibrated in PBS. The conjugated VEGF₁₂₁/rGel was then stored at -80°C until use. For ⁶⁴Cu labeling, ⁶⁴CuCl₂ (148 MBq) was diluted in 300 µl of 0.1 M sodium acetate buffer (pH 5.5) and added to 100 µg of DOTA-VEGF₁₂₁/rGel. The reaction mixture was incubated for 1 h at 40 °C with constant shaking. ⁶⁴Cu-DOTA-VEGF₁₂₁/rGel was then purified by PD-10 column using PBS as the mobile phase. The labeling yield was more than 95% as determined by thin layer chromatography (TLC). About 1.85 MBq (50 µCi) of ⁶⁴Cu-VEGF₁₂₁/rGel (total of 1 µg or less protein) in a volume of 100 µl of PBS was injected into non-tumor-bearing BALB/c mice via the tail vein. At 0.5, 1, 4, 24 and 48 h post-injection, the mice (n = 5/group) were sacrificed. Blood and major organs and tissues were collected and wet-weighed. The radioactivity in the wet whole tissue was read by a γ counter

(Packard). The results were presented as percentage injected dose per gram of tissue (%ID/g). For each mouse, the radioactivity of the tissue samples was calibrated against a known aliquot of the injected tracer and normalized to a mean body mass of each group. Values were expressed as mean $\pm$ SD (n = 5 per group). Assuming similar pharmacokinetics in mice and humans, the activity in organs at different time points post injection was used to calculate single exponential effective half-lives and residence times for each organ [21;22]. Estimated human-absorbed organ doses of ^{64}Cu -DOTA-VEGF₁₂₁/rGel were calculated from the time-activity curves (TACs) derived from the biodistribution data in non-tumor-bearing BALB/c mice with an average weight of 21.3 g using standard female and male human phantoms and the Organ Level Internal Dose Assessment/EXponential Modeling software (OLINDA/EXM; Vanderbilt University, Nashville, TN, USA)[23]. These dose estimates reflect the “area under the curve” for the TAC of %ID/g of tissue for each organ. The Effective dose, which is the sum of the doses to individual organs weighted for both the biological impact of the radiation type and the sensitivity of the organs involved, was then determined for a reference human male using the OLINDA/EXM program [23]. This dose estimates the impact of the absorbed dose and is representative of the overall radiation dose to a subject from PET imaging.

2.5 Determination of Maximal Tolerated Dose and Lethal Dose for VEGF₁₂₁/rGel in Mice

Female BALB/c mice (4–6 weeks old, 5 mice per group) were treated with VEGF₁₂₁/rGel. Based on tissue uptake studies, we devised a QOD X5 i.v. administration schedule to optimize tumor concentration of VEGF₁₂₁/rGel, at doses ranging from 14–40 mg/kg. Survival of mice was monitored every day for three weeks after the final dose.

2.6 Effect of VEGF₁₂₁/rGel on Vascular Permeability

Evans Blue dye was used to examine vascular integrity and permeability to macromolecules following treatment. MDA-MB-231 tumor cells were orthotopically placed in 6–8 week nude mice (3 mice per group). After the tumors had become established (~50 mm³), the mice were injected with VEGF₁₂₁/rGel or saline through a tail vein five times over a 9 day period. The total VEGF₁₂₁/rGel dose was 14 mg/kg. Twenty-four hours after administration of the final dose, 200 μl Evans Blue dye (20 mg/ml in PBS) was injected i.v. and mice were sacrificed 30 minutes later and samples of tissues, tumor and blood were removed, weighed and treated with 0.5 ml DMF for 48 hours. Tumor sizes at the time of sacrifice, as determined by caliper measurements, were not significantly different. Absorbance was measured at 630 nm (Bio-Tek Instruments, Winooski, VT). A linear curve of Evans Blue dye in DMF was established to measure the total amount of dye per tissue.

2.7 Effect of VEGF₁₂₁/rGel on Tumor Hypoxia

MDA-MB-231 tumor cells (2.5×10^6) were orthotopically placed in 6 week nude mice (3 mice per group). After the tumors had become established (15–45 mm³), the mice were injected intraperitoneally with VEGF₁₂₁/rGel (total dose: 11 mg/kg), rGel (4.4 mg/kg, equivalent molar amount) or saline through a tail vein four times over a 7 day period at 100% of the MTD. Eighteen hours after administration of the final dose, mice were injected i.p. with hypoxyprom (1.4 mg; Chemicon International, Inc.). One hour later the mice were sacrificed and tissues harvested. Frozen tumor sections were stained for hypoxia using an

anti-hypoxypore antibody provided in the hypoxypore kit (HP1-100; Chemicon International, Inc.). Necrotic regions were identified using hematoxylin staining. The pixel area of the entire section and the sum of the pixel area of hypoxic/necrotic tumor regions was determined using Metaview software (Universal Imaging Corporation).

2.8 Establishment of NOAEL for VEGF₁₂₁/rGel in BALB/c Mice

Female BALB/c mice (4–6 weeks old, 5 mice per group) were treated with VEGF₁₂₁/rGel (i.v., QOD × 5) at various doses up to the maximum tolerated dose. The vehicle control group consisted of PBS. Seven days after the last injection, animals in the study were sacrificed with carbon dioxide and terminally bled for hematological analyses including a complete blood count (CBC), for clinical chemistry analysis including total bilirubin, phosphorus, AST (SGOT), ALT (SGPT), total protein, albumin, globulin, calcium, sodium, potassium, chloride, alkaline phosphatase, creatinine and BUN, and subjected to a complete necropsy including heart, lungs, spleen, kidneys, liver, etc. All tissues were placed in 10% neutral buffered formalin as the fixative, processed to slides, stained with hematoxylin and eosin, and examined microscopically by the Department of Veterinary Medicine and Surgery of the University of Texas M. D. Anderson Cancer Center.

2.9 Hematological Toxicity of VEGF₁₂₁/rGel in BALB/c Mice

Five female BALB/c mice per group (4–6 weeks old) were injected intravenously every other day (Days 1, 3, 5, 7, 9) for 5 total injections corresponding to 25, 50, 75, and 100 percent of the maximum tolerated dose (MTD). The vehicle control group consisted of PBS. Seven days after the last injection (Day 16), animals in the study were sacrificed with carbon dioxide and terminally bled for hematological analyses including a CBC, which was performed by the Department of Veterinary Medicine and Surgery of the University of Texas M. D. Anderson Cancer Center.

2.10 The Effects of VEGF₁₂₁/rGel on Clinical Chemistry Parameters in BALB/c Mice

Five female BALB/c mice per group (4–6 weeks old) were injected intravenously every other day (Days 1, 3, 5, 7, 9) for total 5 injections corresponding to 25, 50, 75, and 100 percent of the maximum tolerated dose (MTD). The vehicle control group consisted of PBS. Seven days after the last injection (Day 16), animals in the study were sacrificed with carbon dioxide and terminally bled for clinical chemistry analyses including total bilirubin, phosphorus, AST (SGOT), ALT (SGPT), total protein, albumin, globulin, calcium, sodium, potassium, chloride, alkaline phosphatase, creatinine and BUN, which was carried out by the Department of Veterinary Medicine and Surgery of the University of Texas M. D. Anderson Cancer Center.

3. Results and Discussion

3.1 Pharmacokinetic Studies of VEGF₁₂₁/rGel in Mice

The data demonstrated a bi-phasic curve with calculated half-lives of 0.3 and 6 hours for the alpha and beta phases, respectively (Figure 1A). The calculated concentration of VEGF₁₂₁/rGel in plasma at $t = 0$ was 25 $\mu\text{g/ml}$. The immediate apparent volume of distribution of VEGF₁₂₁/rGel was calculated to be 4 ml; approximating the blood volume for a 20 g mouse.

The area under the concentration curve was calculated to be 17.9 $\mu\text{g/ml}$ per hr and the mean residence time for the drug was 3.1 hrs.

3.2 Tissue Distribution of ^{125}I -VEGF₁₂₁/rGel in Nude Mice Bearing MDA-MB-231 Xenografts

We chose orthotopically placed MDA-MB-231 xenografts for biodistribution studies of ^{125}I -VEGF₁₂₁/rGel as MDA-MB-231 cells do not have receptors for VEGF₁₂₁ and this model demonstrates efficient localization of VEGF₁₂₁/rGel to tumor blood vessels [15]. At 48 hours after injection of the radio-labeled protein, the highest uptake was found in the kidneys (tissue:blood ratio of 20.81 ± 3.53). High uptake was also found in the liver (6.02 ± 1.19), spleen (4.59 ± 1.18), and tumor (4.43 ± 0.45) (Figure 1B).

3.3 Biodistribution of ^{64}Cu -DOTA-VEGF₁₂₁/rGel in BALB/c Mice

In order to evaluate the *in vivo* pharmacokinetics of ^{64}Cu -DOTA-VEGF₁₂₁/rGel, we performed a biodistribution assay in BALB/c mice at 0.5, 1, 4, 24 and 48 h post-injection. As shown in Figure 2, ^{64}Cu -DOTA-VEGF₁₂₁/rGel showed relatively high blood retention at early time points (e.g. 26.6 ± 1.73 %ID/g at 30 min p.i.). The circulating ^{64}Cu -DOTA-VEGF₁₂₁/rGel cleared over time and dropped to 11.8 ± 2.83 % ID/g at 60 min after injection and further to 0.82 ± 0.11 % ID/g at 4 hr p.i. The main organs presented high ^{64}Cu -DOTA-VEGF₁₂₁/rGel accumulations include liver, kidneys and spleen, with uptake values of 28.7 ± 2.93 , 40.2 ± 4.03 and 41.5 ± 2.09 %ID/g, respectively, at 4 hr p.i. The uptakes in the brain, muscle, stomach and pancreas were rather low at all time points measured. The distribution of ^{64}Cu -DOTA-VEGF₁₂₁/rGel in the various tissues correlated very well with the distribution of ^{125}I -VEGF₁₂₁/rGel in nude mice, with the exception of spleen, which was relatively higher than observed for ^{125}I -VEGF₁₂₁/rGel. Based on the non-decay-corrected biodistribution data, the human-absorbed dose estimates for normal organs from ^{64}Cu -DOTA-VEGF₁₂₁/rGel were calculated and are as shown in Table 1. The dose-critical organs are liver, kidneys and spleen, where the Effective doses were estimated to be 1.310, 1.590, and 1.890 mGy/MBq, respectively (Table 1). Total body estimated absorbed dose was 0.026 mGy/MBq, with an effective dose of 0.097 mGy/MBq. Overall, our results indicate that micro-dosing studies with ^{64}Cu -DOTA-VEGF₁₂₁/rGel in patients may be useful in predicting response.

3.4 Determination of Maximal Tolerated Dose and Lethal Dose for VEGF₁₂₁/rGel Fusion Toxin in BALB/c Mice

Figure 3 illustrates the survival of mice following administration of VEGF₁₂₁/rGel. All mice treated with saline (control), 14 mg/kg and 17 mg/kg VEGF₁₂₁/rGel survived when treated with the described regimen. One mouse died following the fifth dose of VEGF₁₂₁/rGel at 20 mg/kg, while all the mice administered VEGF₁₂₁/rGel at 30 and 40 mg/kg died by day 14. Accordingly, the LD₅₀ of VEGF₁₂₁/rGel at this schedule in mice is determined to be approximately 25 mg/kg (75 mg/m^2) while the MTD of VEGF₁₂₁/rGel at this schedule is approximately 18 mg/kg (54 mg/m^2). Subsequent *in vivo* efficacy studies have confirmed the safety of the 18 mg/kg dose level.

3.5 Effect of VEGF₁₂₁/rGel on Vascular Permeability

VEGF₁₂₁/rGel increased the vascular permeability of tumor tissue (Figure 4). The concentration of Evans Blue dye was found in the order of tumor > spleen > small intestine > kidneys. Uptake of Evans Blue dye in tumors of VEGF₁₂₁/rGel-treated mice increased by 53% compared to mice treated with saline. While VEGF₁₂₁ can increase vascular permeability [24], it is unlikely to result in the increase observed in these studies. We have previously observed robust damage of tumor vasculature by VEGF₁₂₁/rGel [16], hence the increase in vascular permeability is likely due to direct damage to tumor vascular endothelium.

3.6 VEGF₁₂₁/rGel Treatment Results in Tumor Hypoxia and Necrosis

To understand further the impact of vascular damage to tumor tissue, we investigated the level of hypoxia and necrosis in tumor tissue of mice treated with VEGF₁₂₁/rGel. Hypoxia staining with an anti-hypoxypore antibody revealed hypoxic regions in tumors treated with VEGF₁₂₁/rGel but little or no staining in tumors treated with rGel or PBS (Figure 5A). Quantitation of the effected regions revealed that 10% of the cross-sectional area of VEGF₁₂₁/rGel-treated tumors was hypoxic/necrotic, indicating that significant cell death had occurred in the tumor tissue. Only 3% of the area of PBS-treated tumors ($P = 0.05$; t test, one-sided) and 0% of the area of rGel-treated tumors stained with anti-hypoxypore antibody ($P < 0.11$; t test, one-sided), indicating that the mechanism of antitumor action of VEGF₁₂₁/rGel primarily entails tumor hypoxia secondary to vascular damage (Figure 5B). This data supports our previous observations in a similar breast tumor model that VEGF₁₂₁/rGel efficacy is mediated by induction of tumor hypoxia as determined by ¹⁸F-FMISO uptake [25].

3.7 Establishment of NOAEL for VEGF₁₂₁/rGel in BALB/c Mice

No gross pathology changes were observed with systemic administration of VEGF₁₂₁/rGel. VEGF₁₂₁/rGel caused a very slight decrease in the average terminal body weights in all the treated animals. Apparent dose dependent increases in relative liver and spleen weights were observed (Figure 6). The increase in relative spleen weight correlated with an increase in extramedullary hematopoiesis and lymphoid hyperplasia (follicular cell hyperplasia) observed on microscopic examination. Similarly, the increase in relative liver weights correlated with an increase in extramedullary hematopoiesis observed microscopically in the liver. The relative (to body) kidney weights appeared to be increased, however, this very slight increase paralleled a decrease in the terminal body weights indicating this may be related to a decrease in body weight and not a true organ weight effect. Absence of a microscopic correlate in the kidney sections further supported this is a spurious finding related to the lower body weights in the treated animals. Microscopic lesions in the liver, heart, spleen, skeletal muscle, and bone marrow of BALB/c female mice were observed. The incidence and severity of these lesions appear to be dose-dependent and include effects on hematopoietic cell proliferation in the liver and spleen, where extramedullary hematopoiesis was observed at doses 25% MTD, and the bone marrow, where bone marrow hyperplasia (primarily myeloid) was observed at doses 75% MTD. We also observed follicular cell hyperplasia of the spleen at doses 25% MTD; bone marrow infarction at doses 50%

MTD; cardiomyopathy of the heart at 75 and 100% of the MTD; and skeletal muscle myopathy at 25% MTD. The hematopoietic cell proliferation and follicular cell proliferation were not considered adverse. Microscopic lesions considered adverse were bone marrow infarction, cardiomyopathy, and skeletal muscle myopathy. Skeletal muscle lesions observed at 25% of the MTD were not considered to be clinically important. It should be noted that the only microscopic lesions observed in the 25% MTD total dose group were non-adverse hematopoietic cell proliferation and follicular cell hyperplasia, and a skeletal muscle myopathy that was not clinically important and whose group average grade was less than or equal to the grade of a similar lesion that occurred in a single control animal. None of the mice in the saline controls, 5% MTD and 20% MTD dose groups had consistent skeletal lesions of importance. Thus, the NOAEL was established at 20% of the MTD.

3.8 Hematological Toxicity of VEGF₁₂₁/rGel in BALB/c Mice

The mean hematological parameters in mice after treatment with various doses of VEGF₁₂₁/rGel are shown (Table 2). No test substance-related alterations were noted in hematology parameters with doses of VEGF₁₂₁/rGel up to 100% of the MTD.

3.9 The Effects of VEGF₁₂₁/rGel on Clinical Chemistry Parameters in BALB/c Mice

VEGF₁₂₁/rGel-related increases occurred in AST (aspartate aminotransferase) and ALT (alanine aminotransferase) parameters in a dose-related manner (Table 3). Individual animals in groups treated with VEGF₁₂₁/rGel had a slight increase in these parameters that ranged from 1 – 4 times the upper limit of the reference range for this laboratory. However, the group mean was increased only at the highest dose and was only 2 – 3 times higher than the reference range upper limit for this laboratory indicating a slight elevation at the higher doses. Both of these enzymes are leakage enzymes from several different tissues in mice that include skeletal and cardiac muscle. The elevations of these enzymes are not adverse, but are an indicator of some tissue alteration and serve as surrogates of toxicity.

4. Conclusions

In conclusion, our results show that VEGF₁₂₁/rGel is rapidly cleared from circulation with the primary distribution to the liver, kidney and spleen, and a similar biodistribution profile to ⁶⁴Cu-DOTA-VEGF₁₂₁/rGel. A no adverse effect level (NOAEL) was determined at 20% of the maximum tolerated dose (MTD) of VEGF₁₂₁/rGel. No test substance-related alterations were noted in hematology parameters with doses of VEGF₁₂₁/rGel up to the MTD whereas VEGF₁₂₁/rGel-related increases occurred in AST and ALT parameters in a dose-related manner beyond the NOAEL up to the MTD. VEGF₁₂₁/rGel treatment of tumor-bearing mice indicated an increase in vascular permeability of tumor tissue compared to saline and the primary mechanism of antitumor action as tumor hypoxia secondary to vascular damage.

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Reference List

- Kovtun YV, Goldmacher VS. Cell killing by antibody-drug conjugates. *Cancer Lett.* 2007; 255:232–40. [PubMed: 17553616]
- Senter PD. Potent antibody drug conjugates for cancer therapy. *Curr Opin Chem Biol.* 2009; 13:235–44. [PubMed: 19414278]
- Flavell DJ. Saporin immunotoxins. *Curr Top Microbiol Immunol.* 1998; 234:57–61. [PubMed: 9670612]
- Kwon YM, Li Y, Naik S, Liang JF, Huang Y, Park YJ, Yang VC. The ATTEMPTS delivery systems for macromolecular drugs. *Expert Opin Drug Deliv.* 2008; 5:1255–66. [PubMed: 18976135]
- Li YM, Hall WA. Targeted toxins in brain tumor therapy. *Toxins (Basel).* 2010; 2:2645–62. [PubMed: 22069569]
- Lord MJ, Jolliffe NA, Marsden CJ, Pateman CS, Smith DC, Spooner RA, Watson PD, Roberts LM. Ricin. Mechanisms of cytotoxicity *Toxicol Rev.* 2003; 22:53–64.
- Prince HM, Duvic M, Martin A, Sterry W, Assaf C, Straus DJ. Incidence of spontaneous remission in patients with CD25-positive mycosis fungoides/Sezary syndrome receiving placebo. *J Am Acad Dermatol.* 2012
- Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev.* 2004; 25:581–611. [PubMed: 15294883]
- Fischer C, Mazzone M, Jonckx B, Carmeliet P. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nat Rev Cancer.* 2008; 8:942–56. [PubMed: 19029957]
- Guo S, Colbert LS, Fuller M, Zhang Y, Gonzalez-Perez RR. Vascular endothelial growth factor receptor-2 in breast cancer. *Biochim Biophys Acta.* 2010; 1806:108–21. [PubMed: 20462514]
- Akiyama H, Mohamedali KA, RLES, Kachi S, Shen J, Hatara C, Umeda N, Hackett SF, Aslam S, Krause M, Lai H, Rosenblum MG, Campochiaro PA. Vascular targeting of ocular neovascularization with a vascular endothelial growth factor121/gelonin chimeric protein. *Mol Pharmacol.* 2005; 68:1543–50. [PubMed: 16150930]
- Mohamedali KA, Kedar D, Sweeney P, Kamat A, Davis DW, Eve BY, Huang S, Thorpe PE, Dinney CP, Rosenblum MG. The vascular-targeting fusion toxin VEGF121/rGel inhibits the growth of orthotopic human bladder carcinoma tumors. *Neoplasia.* 2005; 7:912–20. [PubMed: 16242074]
- Mohamedali KA, Poblentz AT, Sikes CR, Navone NM, Thorpe PE, Darnay BG, Rosenblum MG. Inhibition of prostate tumor growth and bone remodeling by the vascular targeting agent VEGF121/rGel. *Cancer Res.* 2006; 66:10919–28. [PubMed: 17108129]
- Mohamedali KA, Li ZG, Starbuck MW, Wan X, Yang J, Kim S, Zhang W, Rosenblum MG, Navone N. Inhibition of prostate cancer osteoblastic progression with VEGF121/rGel, a single agent targeting osteoblasts, osteoclasts, and tumor neovasculature. *Clin Cancer Res.* 2011
- Ran S, Mohamedali KA, Luster TA, Thorpe PE, Rosenblum MG. The vascular-ablative agent VEGF(121)/rGel inhibits pulmonary metastases of MDA-MB-231 breast tumors. *Neoplasia.* 2005; 7:486–96. [PubMed: 15967101]
- Veenendaal LM, Jin H, Ran S, Cheung L, Navone N, Marks JW, Waltenberger J, Thorpe P, Rosenblum MG. In vitro and in vivo studies of a VEGF121/rGelonin chimeric fusion toxin targeting the neovasculature of solid tumors. *Proc Natl Acad Sci U S A.* 2002; 99:7866–71. [PubMed: 12060733]
- Hsu AR, Cai W, Veeravagu A, Mohamedali KA, Chen K, Kim S, Vogel H, Hou LC, Tse V, Rosenblum MG, Chen X. Multimodality molecular imaging of glioblastoma growth inhibition

- with vasculature-targeting fusion toxin VEGF121/rGel. *J Nucl Med.* 2007; 48:445–54. [PubMed: 17332623]
18. Lewis JS, Laforest R, Dehdashti F, Grigsby PW, Welch MJ, Siegel BA. An imaging comparison of ⁶⁴Cu-ATSM and ⁶⁰Cu-ATSM in cancer of the uterine cervix. *J Nucl Med.* 2008; 49:1177–82. [PubMed: 18552145]
19. Vavere AL, Rossin R. *Molecular Imaging of Cancer with Radiolabeled Peptides and PET.* Anticancer Agents Med Chem. 2012
20. Mohamedali KA, Ran S, Gomez-Manzano C, Ramdas L, Xu J, Kim S, Cheung LH, Hittelman WN, Zhang W, Waltenberger J, Thorpe PE, Rosenblum MG. Cytotoxicity of VEGF(121)/rGel on vascular endothelial cells resulting in inhibition of angiogenesis is mediated via VEGFR-2. *BMC Cancer.* 2011; 11:358. [PubMed: 21849059]
21. Lee CL, Wahnische H, Sayre GA, Cho HM, Kim HJ, Hernandez-Pampaloni M, Hawkins RA, Dannoon SF, VanBrocklin HF, Itsara M, Weiss WA, Yang X, Haas-Kogan DA, Matthay KK, Seo Y. Radiation dose estimation using preclinical imaging with ¹²⁴I-metaiodobenzylguanidine (MIBG) PET. *Med Phys.* 2010; 37:4861–7. [PubMed: 20964203]
22. Peng F, Lutsenko S, Sun X, Muzik O. Positron emission tomography of copper metabolism in the Atp7b^{-/-} knock-out mouse model of Wilson's disease. *Mol Imaging Biol.* 2012; 14:70–8. [PubMed: 21327972]
23. Stabin MG, Sparks RB, Crowe E. OLINDA/EXM: the second-generation personal computer software for internal dose assessment in nuclear medicine. *J Nucl Med.* 2005; 46:1023–7. [PubMed: 15937315]
24. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol.* 1991; 5:1806–14. [PubMed: 1791831]
25. Yang M, Gao H, Sun X, Yan Y, Quan Q, Zhang W, Mohamedali KA, Rosenblum MG, Niu G, Chen X. Multiplexed PET probes for imaging breast cancer early response to VEGF/rGel treatment. *Mol Pharm.* 2011; 8:621–8. [PubMed: 21280671]

Figure 1. VEGF₁₂₁/rGel plasma clearance and tissue distribution

(A) Clearance of VEGF₁₂₁/rGel from plasma. The data is plotted as means $\pm$ SEM of the data at each time point. The curve represents the least-squares, best-fit line through these points. (B) Tissue distribution of ¹²⁵I-VEGF₁₂₁/rGel in nude mice bearing MDA-MB-231 xenografts. Nude mice bearing orthotopic MDA-MB-231 tumors were injected intravenously with 1 μ Ci of ¹²⁵I-labeled VEGF₁₂₁/rGel. Data is normalized to tissue:blood ratio $\pm$ SEM.

Figure 2. Biodistribution of ^{64}Cu -DOTA-VEGF₁₂₁/rGel at 0.5, 1, 4, 24 and 48 hr after injection in normal BALB/c mice

Mice were injected intravenously with 1.85 MBq (50 μCi) of ^{64}Cu -DOTA-VEGF₁₂₁/rGel.

Data are presented as mean % ID/g $\pm$ SD (n = 5).

Figure 3. Determination of the maximum tolerated dose and the lethal dose for VEGF₁₂₁/rGel Survival curve for BALB/c mice treated with various doses of VEGF₁₂₁/rGel at a treatment regimen of five doses every other day. Mice treated with a dose up to 17 mg/kg experienced no morbidity. One mouse died following the fifth treatment at 20 mg/kg, and all five mice dead when treated with VEGF₁₂₁/rGel at 30 or 40 mg/kg. Dashed line: Saline, 14 mg/kg and 17 mg/kg; solid line, 20 mg/kg; dotted line, 30 mg/kg and 40 mg/kg.

Figure 4. Tissue distribution of Evans blue dye in nude mice bearing MDA-MB-231 xenografts
The vascular integrity and permeability of blood vessels was examined by intravenous injection of Evans blue dye (20 mg/kg) into tumor-bearing nude mice treated with Saline or VEGF₁₂₁/rGel. Data are presented as nanograms (ng) Evans blue Dye per gram tissue (x 1000) $\pm$ SD.

Figure 5. VEGF₁₂₁/rGel treatment results in hypoxic and necrotic regions in MDA-MB-231 tumors

(A) Representative cross sections of MDA-MB-231 tumors treated with VEGF₁₂₁/rGel (left), rGel (middle), and PBS (right), followed by hypoxyprobe. Hypoxic regions are depicted by arrows. Note that no hypoxic regions were observed in tumors of mice treated with PBS. (B) Quantitation of hypoxic/necrotic regions in MDA-MB-231 tumors.

Figure 6. Group average terminal body weight and relative organ weights as a percent of control VEGF₁₂₁/rGel treatment of female BALB/c mice (n = 5) resulted in a very slight decrease in the overall body weight and a dose related increase in the liver and spleen weights relative to their respective controls. TBW = Terminal Body Weight

Table 1

Human-absorbed radiation doses and Effective doses per organ resulting from administration of 1.85MBq ^{64}Cu -DOTA-VEGF₁₂₁/rGel calculated from mouse biodistribution data (n=5)

Organ	Absorbed dose	Effective dose
Brain	1.30E-02	1.05E-01
Intestine	3.86E-03	3.19E-02
Stomach	1.51E-02	5.59E-02
Heart	9.59E-02	3.54E-01
Kidneys	4.32E-01	1.59E+00
Liver	3.54E-01	1.31E+00
Lung	2.16E-01	7.99E-01
Muscle	1.69E-02	6.26E-02
Red marrow	8.99E-03	3.32E-02
Pancreas	5.24E-02	1.94E-01
Spleen	5.10E-01	1.89E+00
Total Body	2.61E-02	9.67E-02

Doses are expressed as milligray per megabecquerel (mGy/MBq)

Table 2
Hematology group means for BALB/c mice treated with various doses of VEGF₁₂₁/rGel

SEGS %	LY %	MO %	EO %	HGB BA g/dl %	HCT %	WBC 10 ³ /μl	RBC 10 ⁶ /μl	MCV fl	MCH pg	MHC g/dl	Plat 10 ³ /μl
Saline				13.7	40.1	7.5	9.2	43.7	14.9	34.2	605
	18.6	74.0	2.4	2.8	2.2						
25% MTD (4.5 mg/kg)				14.8	41.5	13.4	10.0	41.6	14.8	35.6	608
	18.8	78.4	0.5	1.1	1.2						
50% MTD (9 mg/kg)				11.9	33.7	7.6	7.9	42.7	15.0	35.2	721
	25.1	66.1	3.4	2.4	3.0						
75% MTD (13.5 mg/kg)				11.5	32.6	6.7	7.5	43.3	15.4	35.5	1004
	60.3	3.7	3.8	4.7							
100% MTD (18 mg/kg)				10.9	31.2	6.8	7.3	43.0	15.0	34.8	690
	30.9	9.8	1.1	4.4							
Ref. Range*				12.8–16.4	35.9–48.2	1.4–8.9	7.2–9.8	46.6–52.5	16.1–18.4	33.3–37.5	615–1802
1–35	54.8–92.4	1.5–9.7	1–6.6	0							

* = Reference Range for this laboratory

Abbreviations: HGB, hemoglobin; HCT, hematocrit; WBC, white blood cells; RBC, red blood cells; MCV, mean cell volume; MH, mean hemoglobin per RBC; MHC, mean hemoglobin per hematocrit; Plat, platelets; SEGS, segmented neutrophils; LY, lymphocytes; MO, monocytes; EO, eosinophils; BA, basophils.

Table 3
Clinical chemistry group means for BALB/c mice treated with various doses of VEGF₁₂₁/rGel

ALT IU/L	AP IU/L	T.Prot gm/dl	Alb gm/dl	T. BILI Glob gm/dl	Na ⁺ mEq/L	K ⁺ mEq/L	Cl ⁻ mmol/L	Creat mg/dl	BUN mg/dl	Ca ⁺⁺ mg/dl	PO ₄ mg/dl	AST IU/L
Saline												
124	123	5.5	3.8	0.30	149	8.7	110	0.21	19.0	11.7	8.6	180
				1.7								
25% MTD (4.5 mg/kg)				0.36	149	8.0	110	0.23	19.5	10.4	9.7	381
430	119	5.5	3.7	1.8								
50% MTD (9 mg/kg)				0.26	149	7.8	108	0.19	15.9	10.4	9.1	256
174	115	5.9	3.7	2.2								
75% MTD (13.5 mg/kg)				0.36	149	8.5	110	0.20	17.5	10.3	9.4	542
450	120	5.8	3.6	2.2								
100% MTD (18 mg/kg)				0.35	147	9.4	110	0.17	19.1	10.1	11.3	1030
636	94	5.4	3.5	1.9								
Ref. Range*				0-0.5	146-152	7-11	103-112	0-0.4	18-33	8.9-12.1	8.9-13.6	66-410
0-344	11-224	5.5-6.1	2.9-3.4	2.5-2.8								

* = Reference Range for this laboratory

Abbreviations: T. BILI, total bilirubin; Na⁺, sodium; K⁺, potassium; Cl⁻, chloride; Creat, creatinine; Ca⁺⁺, calcium; PO₄, phosphate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; T.Prot, total protein; Alb, albumin; Glob, globulin.